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VGF (TLQP-62)-induced neurogenesis targets early phase neural progenitor cells in the adult hippocampus and requires glutamate and BDNF signaling☆

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Abstract The neuropeptide VGF (non-acronymic), which has antidepressant-like effects, enhances adult hippocampal neurogenesis as well as synaptic activity and plasticity in the hippocampus, however the interaction between these processes and the mechanism underlying this regulation remain unclear. In this study, we demonstrate that VGF-derived peptide TLQP-62 specifically enhances the generation of early progenitor cells in nestin-GFP mice. Specifically, TLQP-62 significantly increases the number of Type 2a neural progenitor cells (NPCs) while reducing the number of more differentiated Type 3 cells. The effect of TLQP-62 on proliferation rather than differentiation was confirmed using NPCs *in vitro*; TLQP-62 but not scrambled peptide PEHN-62 increases proliferation in a cell line as well as in primary progenitors from adult hippocampus. Moreover, TLQP-62 but not scrambled peptide increases Cyclin D mRNA expression. The proliferation of NPCs induced by TLQP-62 requires synaptic activity, in particular through NMDA and metabotropic glutamate receptors. The activation of glutamate receptors by TLQP-62 activation induces phosphorylation of CaMKII through NMDA receptors and protein kinase D through metabotropic glutamate receptor 5 (mGluR5). Furthermore, pharmacological antagonists to CaMKII and PKD inhibit TLQP-62-induced proliferation of

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NPCs indicating that these signaling molecules downstream of glutamate receptors are essential for the actions of TLQP-62 on neurogenesis. We also show that TLQP-62 gradually activates Brain-Derived Neurotrophic Factor (BDNF)-receptor TrkB *in vitro* and that Trk signaling is required for TLQP-62-induced proliferation of NPCs. Understanding the precise molecular mechanism of how TLQP-62 influences neurogenesis may reveal mechanisms by which VGF-derived peptides act as antidepressant-like agents.

Introduction

Clear evidence now exists that areas in the adult mammalian brain contain pools of neural stem cells that generate new neurons (Grote and Hannan, 2007; Manganas et al., 2007). In the dentate gyrus of the hippocampus, these new neurons mature, become synaptically integrated into the existing circuitry, and contribute towards normal hippocampal function. Adult neurogenesis is comprised of three major processes: proliferation, differentiation and maturation. A multi-step neurogenesis process in the dentate gyrus has been described starting from slow-dividing radial glia-like stem/progenitor cells (type-1) to fast-dividing glial-like, non-radial cells (type 2a) to neuronal determined cells (type 2b) to neuronal determined migratory cells (type 3) and finally to postmitotic mature neurons (Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003; Seri et al., 2004; Steiner et al., 2008). There is evidence that excitatory neuronal activity affects the proliferation of NPCs followed by neuron production and integration of newly born cells into the neuronal network (Deisseroth et al., 2004; Walker et al., 2008). For example, long-term potentiation (LTP) has been shown to enhance proliferation and survival of adult-generated neurons (Bruel-Jungerman et al., 2006). It is known that the process of neurogenesis is dynamic and can be regulated by different physiological, pathological and pharmacological stimuli that also regulate hippocampal synaptic activity. These stimuli include exercise, enriched environments, antidepressants and intrinsic factors (Dranovsky and Hen, 2006; Duman, 2005). However, the specific mechanism by which some of these factors induce neurogenesis is not known.

Intrinsic factors that induce neurogenesis include neurotrophins and neuropeptides, which are released by neuronal activity. Two specific factors, neuropeptide VGF (non-acronymic) and Brain-Derived Neurotrophic Factor (BDNF) have received attention in the context of their contribution to either proliferation or survival of newly born neurons. VGF is a neuronal peptide originally identified as an NGF-responsive gene that is widely expressed in the brain and is involved in maintaining energy balance (Ferri et al., 2011; Levi et al., 2004). *Vgf* is an activity dependent gene and is upregulated in the hippocampus by paradigms that reflect increased activity such as learning (Alder et al., 2003), the induction of LTP (Hevroni et al., 1998), seizures (Snyder et al., 1998), electroconvulsive shock (ECS) (Altar et al., 2004; Newton et al., 2003), voluntary exercise (Hunsberger et al., 2007; Tong et al., 2001) and synaptogenesis (Benson and Salton, 1996; Lombardo et al., 1995). Furthermore, tetrodotoxin (TTX) mediated blockade of retinal activity during the critical period of visual development led to a marked decrease in *Vgf* mRNA levels in the visual cortex, demonstrating that neuronal activity is essential for the induction of VGF expression (Lombardo et al., 1995; Snyder et al., 1998).

We have previously demonstrated that VGF-derived peptide TLQP-62, the C-terminal 62 amino acid peptide enhances neurogenesis of hippocampal cells *in vitro* and *in vivo* (Thakker-Varia et al., 2007). In support of the role of VGF in neurogenesis, a megalencephalic mutant mouse has a two-fold enlarged hippocampus as well as increased levels of VGF (Almgren et al., 2008). Our studies have also established that TLQP-62 enhances synaptic activity of hippocampal neurons acutely in a manner very similar to BDNF and that TLQP-62 is induced following a hippocampal-dependent learning paradigm in rats (Alder et al., 2003). Others have shown that TLQP-62 potentiates synaptic transmission in hippocampal slices in a BDNF-dependent manner (Bozdagi et al., 2008), thus suggesting a close interaction between TLQP-62 and BDNF in the modulation of hippocampal synaptic function. However, the ability of TLQP-62 to regulate TrkB receptor activity and whether the effect of TLQP-62 on synaptic activity is involved in the increase in TLQP-62–induced neurogenesis has not been addressed.

In the present study we have focused on understanding the role of TLQP-62 in the coupling of neurogenesis and synaptic activity. To this end, we have ascertained the precise stage at which the neural progenitor cells are influenced by TLQP-62, and tested the requirement of specific glutamatergic receptors implicated in synaptic activity for the proliferation induced by TLQP-62. In addition, we have explored the downstream signaling pathways that are responsible for TLQP-62-induced neurogenesis via glutamate receptors. Our results also demonstrate that TLQP-62 induces phosphorylation of the TrkB and that this activation appears to be required for TLQP-62-induced proliferation of neural progenitor cells. Revealing the precise molecular mechanism of how TLQP-62 influences neurogenesis will increase our understanding of the pathophysiological mechanisms underlying disorders such as depression, epilepsy, stroke and neurodegenerative diseases in which alterations in adult neurogenesis or neuronal activity has been implicated. In addition, TLQP-62 may represent a way to regulate stem cell proliferation, which has therapeutic potential.

Materials and methods

Peptide treatment *in vivo*

Nestin-GFP transgenic mice on a C57BL/6 background were obtained from Dr. Grigori Enikopolov at Cold Spring Harbor, NY (Mignone et al., 2004). Adult (3–4 month) male mice (~30 g)

were used for *in vivo* experiments. Mice were implanted with a cannula in the lateral ventricle (Alzet Brain Infusion Kit 3, DURECT Corporation, Cupertino, CA) as previously described (Thakker-Varia et al., 2010). Coordinates were anterior–posterior −0.1 mm with respect to bregma; lateral, ±1.0 mm; ventral, −3.0 mm with respect to the surface of the skull (Bartolomucci et al., 2007). Cannulae were secured with Loctite glue. An osmotic minipump (Alzet 2004, 0.25 μl/h) was loaded with saline (4 mice) or TLQP-62 (4 mice) (TLQP-62 C-terminal amidated peptide, Biopeptide Co., San Diego, CA) to deliver 15 μg/day intracerebroventricularly (ICV) for 28 days (Bartolomucci et al., 2007; Siuciak et al., 1997; Ullal et al., 2007; Woodside et al., 2002), connected to the cannula and implanted subcutaneously. A number of neuropeptides and neurotrophins have been shown to be stable for several weeks in the osmotic minipump (Hagg et al., 2005; Larsen et al., 2000; Royo et al., 2006; Sahu, 2002; Shibayama et al., 1998). Two weeks following implantation of cannula and mini-osmotic pump, the mice were injected with 5-ethynyl-2′-deoxyuridine (EdU) (50 mg/kg) (Invitrogen, Grand Island, NY) and perfused with 4% paraformaldehyde (PFA) 2 h later. Correct placement of the cannulae after ICV delivery of TLQP-62 was verified following cryosectioning by visualizing the track of the cannula into the lateral ventricle. Only animals with the correct cannula placement were used for analysis.

Immunohistochemistry

Ten 40 μm sections from each brain were placed in cell filters with PBS 0.5% Triton X100 and 3% BSA. They were then incubated with the Click-iT reaction cocktail (Invitrogen, Grand Island, NY) for 30 min. EdU followed by Click-iT reaction has a clear advantage for tissue sections subjected to multiple antibodies since a third antibody is not required. For triple labeling of TLQP-62 treated mice sections were blocked with 5% Rabbit Serum, 1% BSA, and 0.5% Triton × 100 in PBS. These sections were incubated with primary antibody overnight at 4 °C containing goat polyclonal anti-DCX (1:100, Santa Cruz, Santa Cruz, CA) and chicken polyclonal anti-GFP (1:1000, Invitrogen, Grand Island, NY) in 0.1% Triton in PBS. The sections were then treated with secondary antibody, containing rabbit anti goat 594 (Invitrogen, Grand Island, NY) and rabbit anti-chicken FITC (Millipore, Billerica, MA) at 1:250 in PBS for 2 h. Finally, the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louise, MO) (1:1000). At least thirty cells from the left dentate gyrus of each mouse were counted on a confocal microscope at 40× and determined to be Type 2a (EdU+, Nestin+, DCX−), Type 2b (EdU+, Nestin+, DCX+) or Type 3 (EdU+, Nestin−, DCX+).

Culture of stem cell line derived from adult hippocampus

Neural progenitor cells (NPCs) were kindly provided by the Gage laboratory (Salk Institute). The stem cells were originally isolated from the dentate gyrus of adult Fisher 344 rats. The expanded cultures from single clones were infected with retrovirus to express GFP and selected as previously described (Palmer et al., 1997). They were propagated on poly-L-ornithine (10 μg/ml, Sigma, St. Louis, MO) and laminin (5 μg/ml, Invitrogen, Grand Island, NY) coated plates (polyorn/lam) in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium high glucose (Omega Scientific, Tarzana, CA) containing N2 supplement (Invitrogen, Grand Island, NY), L-glutamine (2 mM), penicillin/streptomycin (100 U/ml) and FGF-2 (20 ng/ml, PeproTech, Rocky Hill, NJ). Cultures of GFP^+ stem cells between passages 15 and 20 were used in this study (Song et al., 2002).

Culture of primary adult hippocampal progenitor cells

P60 CD1 mice were used to isolate the subgranular zone of the hippocampus using a protocol adapted from (Deleyrolle and Reynolds, 2009; Johansson et al., 1999). Tissue was dissociated with papain (20 U/ml), trypsin (1.3 mg/ml), DNAse (0.28 mg/ml), hyaluronic acid (0.7 mg/ml) and kynurenic acid (0.2 mg/ml) followed by mechanical trituration. The cells were plated in growth medium consisting of DMEM/F12 with EGF (20 ng/ml, Peprotech, Rocky Hill, NJ), FGF (20 ng/ml, Peptrotech, Rocky Hill, NJ), 1% B27 (Invitrogen, Grand Island, NY) and antibiotic/antimycotic. Neurospheres were observed within the first week and the medium was changed every 3–4 days. Neurospheres were dissociated into single cells by mechanical trituration and plated on poly-L-ornithine/laminin coated 24 wells as described below.

Proliferation and differentiation studies

NPCs or primary adult hippocampal progenitor cells were plated at 15,000 cells/cm^2 on poly-L-ornithine/laminin coated dishes in DMEM/F12 medium containing N2 supplement as described above with the exclusion of FGF-2 and EGF. In some experiments, NPCs at 15,000 cells/cm^2 were co-cultured on hippocampal primary neurons (10 days *in vitro*) in serum-free co-culture medium containing DMEM, N2 supplement, sodium pyruvate (1 mM), glucose (0.2 M), L-glutamine (2 mM), ovalbumin (0.1%), and penn/strep. NPCs cultured on polyorn/lam coated dishes or on hippocampal cell dishes were treated with either vehicle, TLQP-62 (3 μM), scrambled peptide PEHN-62 (3 μM) or KCl (20 mM). The dose of TLQP-62 was selected based on our previous *in vitro* studies (Thakker-Varia et al., 2007). For proliferation studies, 3 days after plating, BrdU (10 μM, Sigma, St. Louis, MO) or EdU (10 μM, Invitrogen, Grand Island, NY) was added for 2 h followed by fixation with 4% paraformaldehyde. For differentiation studies, the medium and factors were replaced after 4 days and the cells were fixed after 7 days.

Hippocampal neuronal cultures

E18 (embryonic day 18) hippocampi were obtained from timed-pregnant Sprague Dawley rats (Charles River, Wilmington, MA) killed by CO_2 asphyxiation in accordance with institutional guidelines for care and use of animals. Pooled tissue from each litter was mechanically triturated in Eagle's Minimum Essential Medium (MEM) with glucose and 7.5% fetal bovine serum and plated on poly-D-lysine-coated petri dishes at 350,000 cells/35 mm dish. Cultures were maintained in serum-free Neurobasal medium containing B27 (Invitrogen, Grand Island, NY) and glutamine (SFM) at 37 °C in a 95% air/5% CO_2 humidified incubator as previously described

(Thakker-Varia et al., 2001) and contained virtually pure neurons.

Inhibitor treatments

Cells were pretreated with inhibitors for 30 min prior to the addition of TLQP-62 (3 μM). Inhibitors used were as follows: tetrodotoxin (TTX) (1 μM, Sigma, St. Louis, MO), AP5 (50 μM, Sigma, St. Louis, MO), 2-Methyl-6-(phenylethynyl)pyridine (MPEP) (100 μM, Sigma, St. Louis, MO) bicuculline (20 μM, Sigma, St. Louis, MO), nifedipine (10 μM, Sigma, St. Louis, MO), w-conotoxin GVIA (1 μM, Millipore, Billerica, MA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μM, Sigma, St. Louis, MO), KN-93 (5 μM, Sigma, St. Louis), GF109203x (10 μM, Tocris Bioscience, Minneapolis, MN), and K252a (200 nM, Millipore, Billerica, MA).

Immunocytochemistry

For the proliferation studies on NPCs, cells were blocked with 30% goat serum in PBS/0.3% Triton X-100. Chicken anti-GFP (1:1000. Invitrogen, Grand Island, NY) was applied overnight at 4 °C followed by rabbit anti-chicken FITC (1:500, Millipore, Billerica, MA) for 1 h. The cells were then fixed again with 4% paraformaldehyde, treated for 30 min with 2 N HCl, followed by anti-BrdU (1:100, Becton Dickinson, San Jose, CA) for 1 h and Alexa Fluor 594 goat anti-mouse (1:1000, Invitrogen, Grand Island, NY) for 1 h. EdU staining for primary adult hippocampal progenitor cells followed the manufacturers recommended protocol for the Click-It reaction (Invitrogen, Grand Island, NY) followed by DAPI to label nuclei. For the differentiation studies, cells were blocked in 2% goat serum in PBS/0.1% Triton X-100 followed by MAP2ab (1:1000, Sigma, St. Louis, MO) overnight at 4 °C and Alexa Fluor 594 goat anti-mouse (1:1000) for 1 h. The dishes were then coverslipped with Flouromount G (Southern Biotech, Birmingham, AL) and examined using a Zeiss Axiovert 200 M microscope at 40× magnification. Approximately 200 GFP+ cells were counted on every dish and the percent of those cells that also expressed BrdU or MAP2 was quantified. The data for each set were normalized to the control for the set. For receptor expression, anti-TrkB (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-NMDA (1:100), anti-GluR2/3 (1:100) and anti-mGluR5 (1:500) from Millipore (Billerica, MA) were used overnight at 4 °C followed by Alexa Fluor 594 goat anti-mouse (1:1000).

Real time RT-PCR

NPCs were grown in N2 medium without bFGF and treated with vehicle, TLQP-62 (3 μM) or scrambled peptide PHEN-62 (3 μM) for 3 days. Total RNA was isolated from NPCs using Trizol Reagent according to the manufacturer's protocol (Invitrogen, Grand Island, NY). RNA was subjected to RQ1 DNAse treatment (Promega, Grand Island, NY) and phenol chloroform extraction to remove genomic DNA. 100 μl of cDNA was prepared from 2 μg RNA using random primers and Superscript II reverse transcriptase (Invitrogen, Grand Island, NY). 25 μl PCR reactions were then carried out using gene specific primers designed by Primer Express software and SYBR Green (Applied Biosystems 7000 Sequence Detection System, Foster City, CA). Duplicate wells were included for each condition and primer pair. Primers to rodent *Cyclin D1* and *D2* were selected using Primer Express software (Applied Biosystems). Primers specific to the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* were used as an internal control. Data analysis was performed according to the protocol provided by Applied Biosystems.

Western blot

Hippocampal neurons were solubilized in RIPA buffer and protein content determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Forty micrograms of protein was denatured with NuPAGE Sample Reducing Agent (Invitrogen, Carlsbad, CA) followed by separation on a 10% BisTris NuPAGE gel (Invitrogen, Carlsbad, CA) and transfer to PVDF membrane. Membranes were probed with anti-phospho-CaMKII (1:1000, Abcam, Cambridge, MA), anti-CaMKII (1:10,000, Abcam, Cambridge, MA), anti-phospho-PKD (1:500, Millipore, Billerica, MA) or anti-PKC mu (1:1,000, Abcam, Cambridge, MA). Membranes were washed, followed by 1 h incubation with donkey anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (1:5000, GE Healthcare, Pittsburgh, PA) at room temperature. Proteins were detected using enhanced chemiluminescence (PerkinElmer, Waltham, MA). Levels of the immunopositive bands were quantified densitometrically using Quantity One V 4.2.1 software on a GelDoc 2000 (BioRad, Hercules, CA) (Thakker-Varia et al., 2001).

Phospho-Trk ELISA

An enzyme-linked immunosorbent assay (ELISA) method was used to measure the level of phosphorylated Trk receptors from primary neurons as described (Rantamaki et al., 2011). After TLQP-62 and BDNF treatments, the medium was removed and the cells were lysed with cold lysis buffer. Following 30 min incubation on ice cell lysate was transferred to pre-coated (sc-11-R, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA; O/N at 4 °C) and pre-blocked (2% BSA/PBS-T; 2 h at room temperature) white 96-well Optiplate™ (PerkinElmer) plates. The plates were incubated overnight at 4 °C and the wells washed with PBS-T and anti-phosphotyrosine antibody was added to the wells (biotinylated PY20, AbD Serotec, Raleigh, NC 1:1000 in 2% BSA/PBS-T; O/N at 4 °C). Following sequential washes and HRP-coupled tertiary antibody incubations (Streptavidin-HRP, 1:10000 in 2% BSA/PBS-T; overnight at 4 °C) ECL substrate (Pierce, Rockford, IL) was added to the wells and luminescence measured after 5 min with Varioskan Flash (Thermo Fisher Scientific, Pittsburgh, PA) plate reader.

Statistical analysis

Statview software was used for analysis of all data. Data were analyzed using two-tailed Student t-test or ANOVA followed by Fishers PLSD post hoc test for multiple comparisons. $p < 0.05$ is considered significant.

Results

Determination of phases of neurogenesis

Our earlier studies demonstrate that the neuropeptide TLQP-62 enhances BrdU incorporation both *in vitro* and *in vivo* into cells that eventually differentiate into neurons (Thakker-Varia et al., 2007). However, the exact stage of neurogenesis at which TLQP-62 acts is not known. We have now taken advantage of a very precise staging scheme based on marker expression (Steiner et al., 2008) to identify which progenitor population is affected by TLQP-62. We employed transgenic mice whose precursor cells in the hippocampus are labeled with GFP driven by the Nestin promoter (Nestin-GFP). The regulatory elements of the *nestin* gene direct reporter gene expression to the neuroepithelium of the embryo and to stem and progenitor cells of the adult brain (Encinas et al., 2006). TLQP-62 peptide (15 μg/day) or saline vehicle was infused intracerebroventricularly (ICV) for 2 weeks by mini-osmotic pump into adult Nestin-GFP transgenic mice and the proliferating cells identified by an EdU pulse. EdU is a thymidine analog, and EdU labeling and detection is very sensitive and specific as it is based on Click-iT (Invitrogen) chemistry, which does not require any other manipulations used for BrdU labeling. Co-labeling of cells with EdU and different stage-specific antibodies allowed us to delineate the phases of neurogenesis influenced by TLQP-62. EdU was co-localized with Nestin-GFP and doublecortin (DCX). We quantitated the proportion of Type 2a (EdU+, Nestin+, DCX−), Type 2b (EdU+, Nestin+, DCX+), and Type 3 (EdU+, Nestin−, DCX+) cells in the dentate gyrus of the hippocampus (Figs. 1A and B). TLQP-62 infused mice exhibited a significant increase in the proportion of cells that are Type 2a and a decrease in the proportion of cells that are Type 3 relative to the saline infused mice (Fig. 1C). These data suggest that TLQP-62 promotes early phases of neurogenesis when proliferation is occurring and inhibits differentiation. This is similar to what has been reported following antidepressant treatment and voluntary exercise (Boku et al., 2011; Encinas et al., 2006; Kronenberg et al., 2003; Steiner et al., 2008).

To study the mechanism by which TLQP-62 enhances proliferation in progenitor cells, we used an adult rodent neural progenitor cell (NPC) line. NPCs have been shown to retain stem cell phenotypes *in vitro* and behave identically to endogenous NPCs when implanted into the intact hippocampus (Palmer et al., 1997). NPCs labeled with GFP, were grown either on a polyornithine/laminin coated surface or on hippocampal cultures derived from E18 rat embryos to mimic a more *in vivo* type of environment (Alder et al., 2003; Song et al., 2002). To determine the effect of TLQP-62 on proliferation, TLQP-62 (3 μM) was added to the NPCs after mitogen withdrawal for 72 h followed by BrdU (10 μM) for 2 h to label cells undergoing DNA synthesis. KCl (20 mM) which directly stimulates synaptic activity was used for comparison as a stimulant of synaptic activity. All data were normalized to PBS vehicle. We observed that TLQP-62 increases the number of NPCs that are positive for BrdU both on a polyornithine/laminin coated substrate (vehicle 34.85 ± 4.58, TLQP-62 51.03 ± 5.35, % BrdU+) as well as on a bed of hippocampal neurons (vehicle 34.41 ± 5.39, TLQP-62 49.70 ± 7.04, % BrdU+) to approximately the same degree. KCl, on the other hand had no effect on the number of proliferating cells on either substrate (Fig. 2B).

To assess if TLQP-62 affects the number of NPCs differentiating into neurons, immunofluorescence of Microtubule Associated Protein 2 (MAP2), which specifically labels dendrites, was quantitated after 7 days of TLQP-62 or vehicle treatment. TLQP-62 does not affect the NPCs that express the differentiation marker MAP2 on polyornithine/laminin (vehicle 37.39 ± 6.26% MAP2+, TLQP-62 42.53 ± 6.98% MAP2+) or hippocampal neuronal substrate relative to control (vehicle 10.31 ± 1.89% MAP2+, TLQP-62 12.56 ± 2.53% MAP2+). KCl treatment did enhance the number of MAP2 positive cells on the polyornithin/laminin coated dishes but showed an inhibitory trend on MAP2+ cells on a hippocampal bed (Fig. 2E), perhaps due to excitotoxicity. Previous studies have shown that KCl does not affect adult NPC progeny

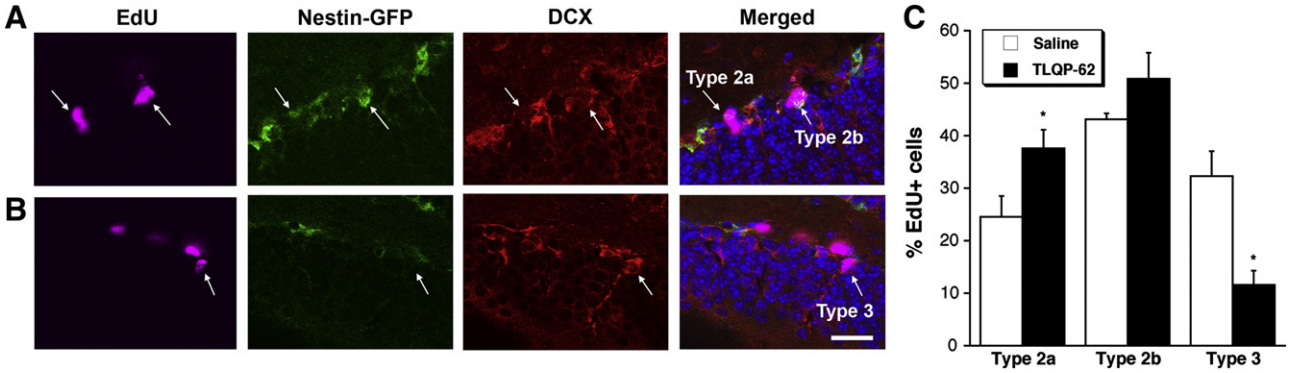

Figure 1 TLQP-62 promotes proportion of early rather than later phase neural progenitors *in vivo*. *A*, Immunohistochemistry of Nestin-GFP adult mice injected with EdU (50 mg/kg) for 2 h. Type 2a neuron in the SGZ indicated by arrow is EdU+ (pink), Nestin+ (green) and DCX-, and Type 2b neuron in SGZ indicated by arrow is EdU+ (pink), Nestin+ (green) and DCX+ (red). *B*, Type 3 neuron in the SGZ indicated by arrow is EdU+ (pink), Nestin− and DCX+ (red). *C*, Quantitation of proportion of EdU+ cells for Types 2a, 2b and 3. Bars represent average percent ±SEM. Proportion of Type 2a NPCs from TLQP-62-treated dentate gyri increases relative to Saline. Proportion of EdU+ cells for Type 3 NPCs from TLQP-62-treated dentate gyri is significantly lower relative to the saline condition, n = 30–35 EdU+ cells per brain, 4 brains per condition, *$p < 0.05$, *t*-test.

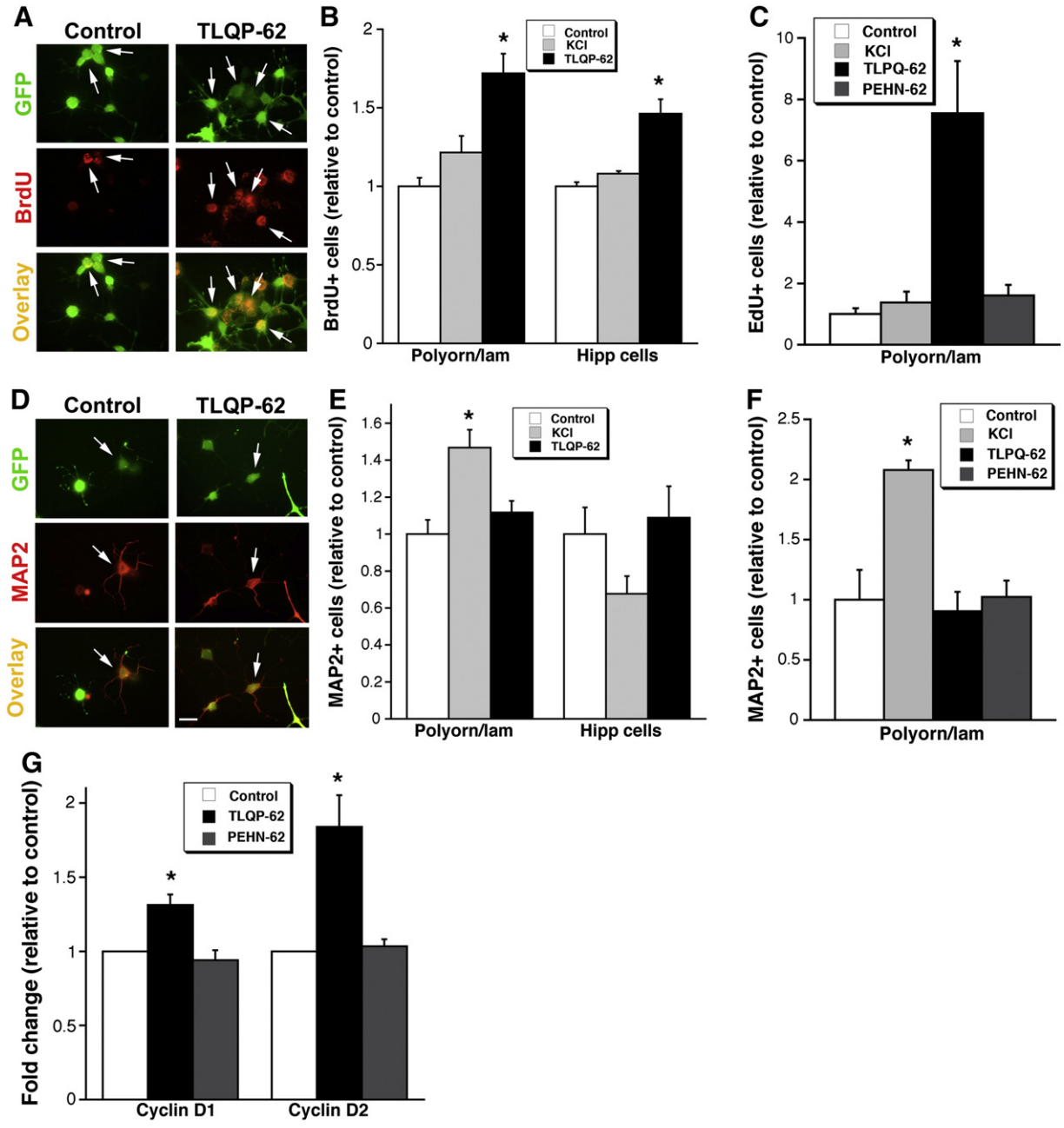

Figure 2 TLQP-62 enhances proliferation but not differentiation of neural progenitor cells *in vitro* and increases *Cyclin D1* and *D2* mRNA levels. *A*, Representative images of GFP-expressing NPCs grown on polyornithine/laminin (polyorn/lam) coated dishes treated with vehicle or TLQP-62 (3 μM) for 3 days followed by BrdU (10 μM) for 2 h. Arrows represent double-labeled cells. *B*, Quantitation of BrdU+/GFP+ NPCs grown on polyorn/lam or hippocampal neurons (hipp cells). KCl (20 mM) is used as a control. *C*, Quantitation of EdU+/DAPI+ primary adult hippocampal progenitor cells grown on polyorn/lam. *D*, Representative images of GFP-expressing NPCs grown on polyorn/lam coated dishes treated with vehicle or TLQP-62 (3 μM) for 7 days followed by MAP2 staining. Arrows represent double-labeled cells. Scale bar for A and D = 30 μM. *E*, Quantitation of MAP2+/GFP + cells grown on polyorn/lam or hipp cells. *F*, Quantitation of EdU+/DAPI + primary adult hippocampal progenitor cells grown on polyorn/lam. Bars represent average ± SEM normalized to the vehicle control for each experiment (n = at least 200 cells/dish, 5,6 dishes/condition for NPCs and 8,9 wells/condition for primary adult hippocampal progenitors). *G*, Average *Cyclin D1* and *D2* gene expression in NPCs treated with scrambled peptide (PEHN-62) and TLQP-62 (3 μM) for 3 days. All samples were first normalized to GAPDH and then represented as an average ratio of vehicle control ± SEM * (n = 3). *$p < 0.05$ relative to control, ANOVA Fishers PLSD.

proliferation, survival, or apoptosis but rather promotes neuronal differentiation (Deisseroth et al., 2004). Our findings of an effect of KCl on MAP2-positive cells but not on BrdU positive cells are consistent with the findings of these studies. Our *in vitro* results are also in parallel with the *in vivo* studies, which indicates that TLQP-62 promotes proliferation and the early phase of neurogenesis rather than the later differentiating phase.

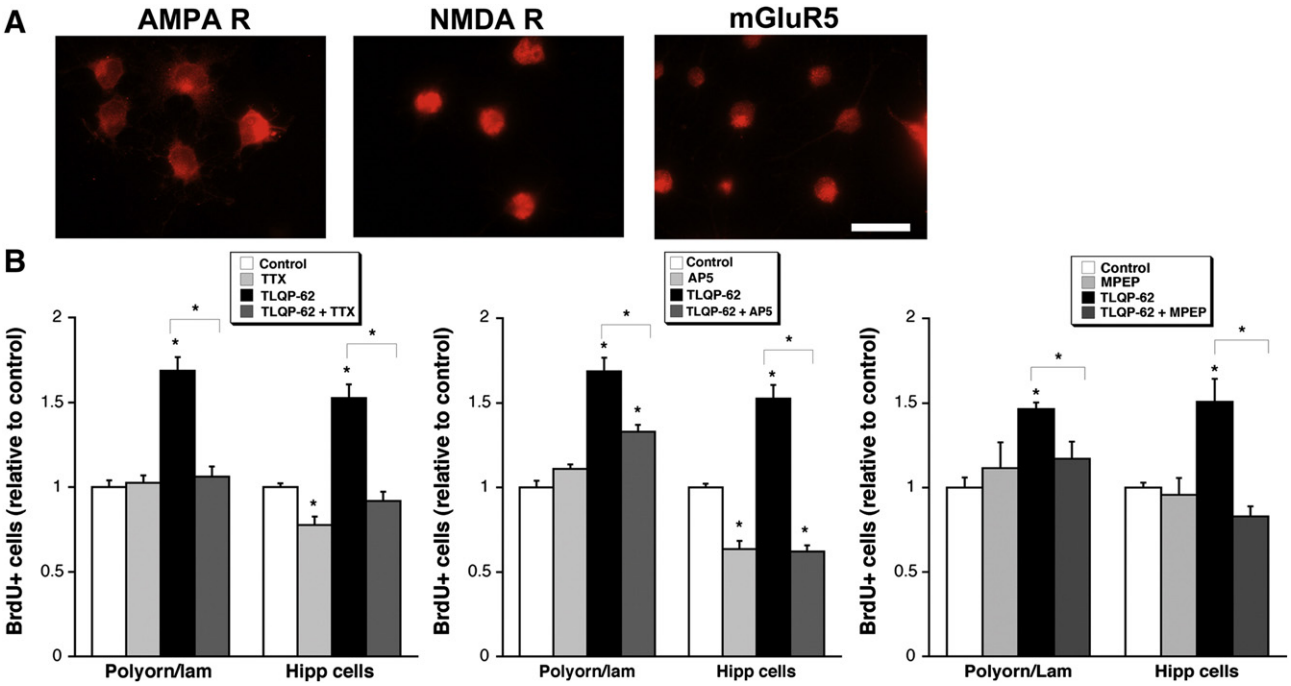

Figure 3 TLQP-62-induced proliferation requires Na^+ channels, NMDA receptors and mGluR5. *A*, Representative images of NPCs *in vitro* immunostained for AMPA receptor, NMDA receptor, and metabotropic glutamate receptor 5. Scale bar = 30 μM. *B*, NPCs grown on polyornithine/laminin (polyorn/lam) coated dishes or hippocampal cells (hipp cells) were pretreated with TTX (1 μM), AP5 (50 μM), or MPEP (100 μM) for 30 min prior to the addition of TLQP-62 (3 μM). After 3 days, cells were treated with BrdU (10 μM) for 2 h followed by fixation and immunocytochemistry for GFP and BrdU. Bars represent average number BrdU+/GFP+ cells normalized to the vehicle control for each experiment ±SEM (n = 9–12 dishes/condition, 200 cells/dish). *$p < 0.05$ relative to control, * with bracket represents significant difference between TLQP-62 and TLQP-62 plus inhibitor, ANOVA Fishers PLSD.

To explore the effect of TLQP-62 on primary neural stem cells, adult hippocampal progenitor cells were acutely isolated from mouse dentate gyrus and grown *in vitro*. Cells were initially grown as neurospheres and then dissociated and plated on polyornithine/laminin coated wells to assess proliferation. TLQP-62 treatment (3 μM) for 3 days had a robust effect on proliferation of those progenitor cells relative to vehicle control or a scrambled control peptide (PEHN-62) as shown by EdU (10 μM) incorporation for 2 h (vehicle 2.25 ± 0.44% BrdU+, PEHN-62 3.73 ± 0.82, TLQP-62 15.08 ± 2.14% BrdU+). Similar to the NPC line, primary adult hippocampal progenitor cells did not increase proliferation in response to KCl (Fig. 2C). Moreover, there was no increase in MAP2+ cells when the primary hippocampal progenitor cells were treated TLQP-62 or PEHN-62 for 7 days (vehicle 21.29 ± 3.64% MAP2+, PEHN-62 22.77 ± 4.60% MAP2+, TLQP-62 20.78 ± 3.62% MAP2+) although primary neural progenitor cells did show increased MAP2 expression in response to KCl (Fig. 2F). These findings support the conclusion that TLQP-62 promotes proliferation but not differentiation of hippocampal progenitor cells isolated from the adult hippocampus.

The effect of TLQP-62 on cyclin gene expression was examined to further explore the role of the neuropeptide in proliferation. NPCs were treated for 3 days with TLQP-62 or scrambled control peptide PEHN-62 and subjected to real time RT-PCR for *Cyclin D1 and D2*. D-type cyclins control the transition from G1 to S (Sherr, 1994). They become synthesized in response to mitogens and their expression rapidly declines when mitogens are withdrawn (Matsushime et al., 1994). The levels of *Cyclin D1* and *D2* mRNA were both increased in TLQP-62 treated NPCs when compared to cells treated with the scrambled peptide PEHN-62 or vehicle (Fig. 2G). Thus TLQP-62 appears to enhance the expression of genes, which regulate the transition from G1 to S phase and promote proliferation.

Requirement for synaptic activity in TLQP-62-induced neurogenesis

We have previously shown that TLQP-62 enhances synaptic activity (Alder et al., 2003). However, the requirement of activity for the effects of TLQP-62 on neurogenesis has not been studied. To examine this question, NPCs were grown *in vitro* in the presence of TLQP-62 plus selected pharmacological agents. It has been demonstrated that brief excitation applied to actively proliferating NPCs is sufficient for excitation-neurogenesis coupling. Furthermore, NPCs do express Na^+ channels and KCl-induced neurogenesis has been shown to be blocked by TTX (Deisseroth et al., 2004). To confirm that the NPCs express neurotransmitter receptors, immunocytochemistry was performed and we confirmed that all the cells expressed AMPA, NMDA and mGluR5 receptors (Fig. 3A). To determine if TLQP-62 requires activity to induce neurogenesis, cultures were pretreated with inhibitors for 30 min prior to TLQP-62 exposure. The following specific antagonists were used: Na^+ channel (TTX, 1 μM), Ca^{2+} channel (Nifedipine, 10 μM), NMDA receptor (AP5,

50 μM), AMPA receptor (CNQX, 1 nmol/0.5 μl), metabotropic glutamate receptor (MPEP, 1 mM), $GABA_A$ receptor (bicuculline, 10 μM). TLQP-62 significantly increased the number of BrdU+ cells relative to vehicle. Our results indicate that the inhibitors alone had no significant effect on the number of BrdU+ cells except for TTX on a bed of hippocampal neurons. However, pretreatment of NPCs with TTX, AP5 and MPEP prior to addition of TLQP-62 all significantly reduced the number of BrdU+ NPCs relative to TLQP-62 alone (Fig. 3B). This suggests that NMDA receptors and mGluR5 are required for TLQP-62 induced neurogenesis. Several other synaptic activity inhibitors including the L-type voltage dependent Ca^{2+} channel antagonist nifedipine, the N-type Ca^{2+} channel antagonist w-conotoxin, the GABA receptor antagonist bicuculline, and the AMPA/kainate receptor inhibitor CNQX had no effect on TLQP-62-induced proliferation (Supp Fig. 1), indicating that these molecules are not involved in TLQP-62-induced neurogenesis.

Signaling pathways downstream of TLQP-62-induced neurogenesis

We have explored the downstream signaling events activated by TLQP-62 to mediate its actions on neurogenesis. We first determined if TLQP-62 activates signaling molecules in hippocampal cells downstream of the two glutamate receptors implicated in TLQP-62-induced proliferation, NMDA and mGluR5. CaMKII can associate with NMDA receptors and ultimately sustain its activation state (Bayer et al., 2001). Moreover, activation of mGluR5 induces phosphorylation of protein kinase D (PKD) in hippocampal neurons (Krueger et al., 2010). Western blot analysis was performed on hippocampal cells pretreated with an antagonist to NMDA receptors, AP5 (50 μM) or an antagonist to mGluR5, MPEP (100 μM) for 30 min prior to addition of TLQP-62 (3 μM) for 15 min. Activation of CaMKII and PKD was examined using phospho-specific antibodies. Quantitation of the Western blots indicates that TLQP-62 enhances phosphorylation of CaMKII and that this activation can be blocked by AP5, suggesting that CaMKII is a downstream target of NMDA receptors activated by TLQP-62 (Fig. 4). Similarly, we have demonstrated that TLQP-62 increases phosphorylation of PKD relative to control and that this activation can be significantly reduced by the mGluR5 receptor antagonist, indicating that PKD is downstream of mGluR5 receptors activated by TLQP-62 (Fig. 4). The inhibitors alone had no significant effect on phosphorylation of the kinases.

To further determine the role of these signaling pathways in TLQP-62-induced neurogenesis, we used inhibitors to CaMKII and PKD. Previous research demonstrated KN-93's potential to inhibit CaMKII, preventing glutamate-induced miniature excitatory postsynaptic currents, a process rescued through the application of additional CaMKII (Ninan and Arancio, 2004).

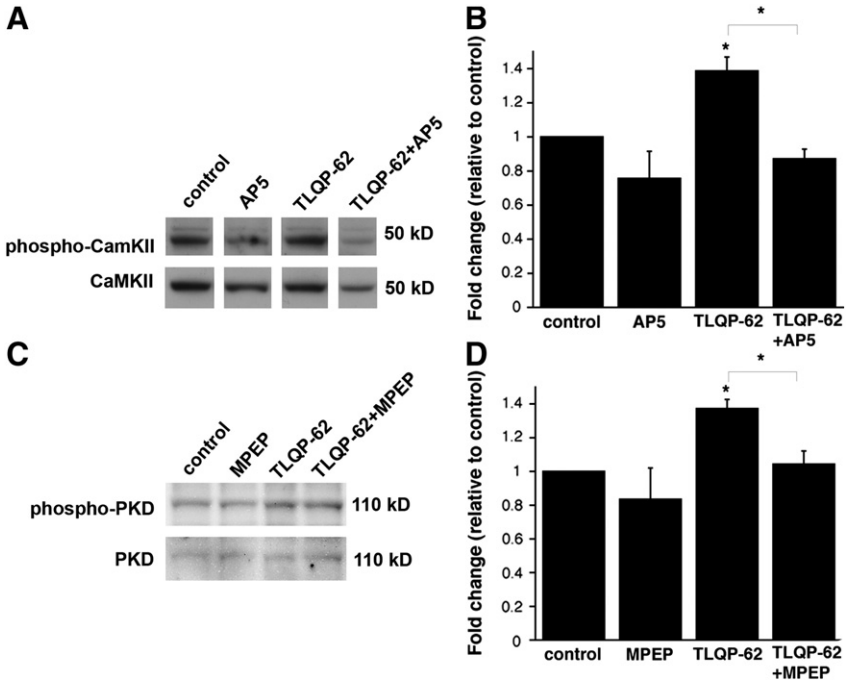

Figure 4 TLQP-62 activates CaMKII *via* NMDAR and PKD *via* mGluR5. *A*, Representative Western blot of hippocampal cultures (10 div) that had been pretreated with AP5 (50 μM) or vehicle for 30 min followed by TLQP-62 (3 μM) for 15 min. Protein lysates were run on a 10% BisTris gel and the membrane probed for phospho-CaMKII followed by CaMKII for a loading control. *B*, Quantitation of CaMKII phosphorylation. Bars represent average protein expression relative to control ± SEM (n = 3). *C*, Representative Western blot of hippocampal cultures (10 div) that had been pretreated with MPEP (100 μM) or vehicle for 30 min followed by TLQP-62 (3 μM) for 15 min. Protein lysates were run on a 10% BisTris gel and the membrane probed for phospho-PKD followed by PKC mu (PKD) for a loading control. *D*, Quantitation of PKD phosphorylation. Bars represent average protein expression relative to control ± SEM (n = 3). *$p < 0.05$ relative to control, * with bracket represents significant difference between TLQP-62 and TLQP-62 plus inhibitor, ANOVA Fishers PLSD.

Similarly, GF109203x has been shown to be a potent inhibitor of PKD in primary cells (Jadali and Ghazizadeh, 2010; Van Lint et al., 1998). NPCs were pretreated for 30 min with either KN93 (5 μM) to prevent CaMKII activation or with GF109203x (10 μM) to inhibit PKD activation. TLQP-62 (3 μM) when then added to these NPCs for 72 h followed by addition of BrdU (10 μM) for 2 h. Our data indicate that the TLQP-62-induced increase in BrdU+ cells is significantly prevented by treatment with either KN93 or GF109203x (Fig. 5). The inhibitors alone did not have any effect on cell proliferation as indicated by the number of BrdU+ cells. The finding that TLQP-62 no longer enhances neurogenesis in the presence of those pharmacological agents that inhibit CaMKII and PKD suggests that signaling through glutamate receptors via CaMKII and PKD is required for the effects of TLQP-62 on neurogenesis.

Role of BDNF signaling in TLQP-62-induced neurogenesis

Previous studies suggest that TLQP-62 effects on synaptic plasticity requires TrkB activation and may promote BDNF release (Bozdagi et al., 2008). Although two receptors for TLQP-21, both of which are complement receptors, have recently been described (Chen et al., 2013; Hannedouche et al., 2013), the receptor for TLQP-62 has not yet been identified. We therefore began to explore the interaction between TLQP-62-induced neurogenesis and TrkB. We first demonstrated that TrkB is expressed on all NPCs (Fig. 6A). To determine whether Trk is required for the effect of TLQP-62 on proliferation of NPCs, we used the K252a (200nM), a broad-spectrum kinase inhibitor commonly used to block BDNF-induced Trk phosphorylation. Pretreatment with K252a alone for 30 min had no effect on BrdU incorporation. However, NPCs pretreated with K252a followed by TLQP-62 (3 μM) for 3 days had significantly reduced incorporation of BrdU relative to cells treated with TLQP-62 alone (Fig. 6B). These data suggest that Trk receptors are helping to mediate the actions of TLQP-62. To determine the mechanism by which TLQP-62 interacts with Trk receptors, we quantitated TrkB phosphorylation by TLQP-62 using an ELISA approach (Rantamaki et al., 2011). Primary neurons treated with TLQP-62 (3 μM) for either 2 or 6 h did not show induction of TrkB activation. However, cells treated with TLQP-62 for 24 h exhibited significantly higher TrkB phosphorylation relative to vehicle-treated control cells. Cells treated with a higher concentration of TLQP-62 (6 μM) for 2 h also showed a significant increase in TrkB activation relative to vehicle (control 100.00 ± 2.53, TLQP-62 111.80 ± 3.30 fold induction, $n = 20$, $p < 0.05$, *t*-test). As a positive control, cells were treated with BDNF (5 ng/ml) and TrkB phosphorylation was detected within 15 min. TLQP-62 treatment (3 μM) for less than 2 h did not show any induction of phospho-TrkB (data not shown). These findings demonstrate that the effect of TLQP-62 on TrkB requires an extended period of time relative to BDNF and suggest that the effect of TLQP-62 on TrkB phosphorylation is much less robust than that of BDNF.

Discussion

This study demonstrates that the neuropeptide TLQP-62 enhances the generation of early progenitor cells in the dentate gyrus and that mGluR5 and NMDA receptors are required for TLQP-62-induced proliferation of NPCs. The signaling cascades activated by TLQP-62 downstream of the glutamate receptors include PKD and CaMKII, which are necessary for TLQP-62-mediated proliferation of NPCs. We also investigated the relationship between TLQP-62 and BDNF signaling and demonstrate not only that TLQP-62 promotes TrkB phosphorylation but also that Trk activation is required for TLQP-62-induced proliferation of NPCs.

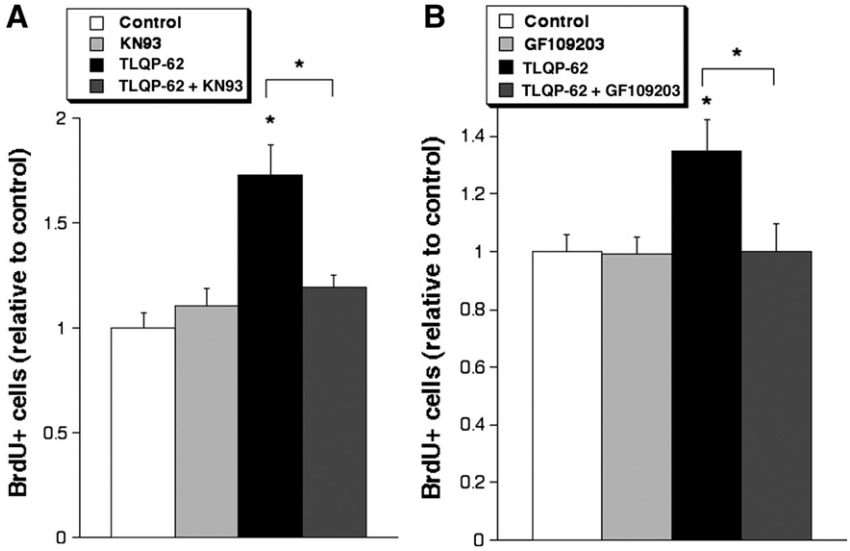

Figure 5 TLQP-62-induced proliferation requires CaMKII and PKD. NPCs grown on polyorn/lam coated dishes were pretreated with *A*, KN-93 (5 μM), or *B*, GF109203x (10 μM) for 30 min prior to the addition of TLQP-62 (3 μM). After 3 days, cells were treated with BrdU (10 μM) for 2 h followed by fixation and immunocytochemistry for GFP and BrdU. Bars represent average number BrdU+/GFP+ cells normalized to the vehicle control for each experiment ±SEM (n = 9 dishes/condition, 200 cells/dish). *$p < 0.05$ relative to control, ANOVA Fishers PLSD.

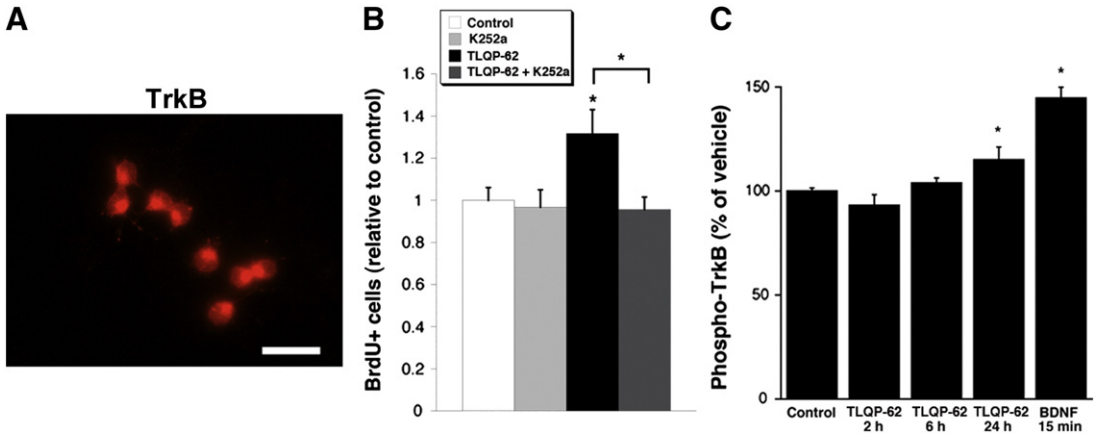

Figure 6 Trk is required for TLQP-62-induced proliferation of neural progenitor cells *in vitro*. *A*, Representative image of NPCs *in vitro* immunostained for TrkB receptor. *B*, NPCs grown on polyorn/lam coated dishes were pretreated with K252A (200nM) followed by BrdU (10 μM) for 2 h. Bars represent average number BrdU+/GFP+ cells normalized to the vehicle control for each experiment ±SEM (n = 200 cells/dish, 9–12 dishes/condition). *p < 0.05 relative to control, * with bracket represents significant difference between TLQP-62 and TLQP-62 plus inhibitor, ANOVA Fishers PLSD. *C*, Neurons (21 div) were treated with TLQP-62 (3 μM) or BDNF (5 ng/ml) for various times indicated and TrkB phosphorylation measured by phosphor-TrkB ELISA. Data is presented as percentage of control ± SEM (n = 5–18). *p < 0.05 relative to control t-test.

Together these findings lend insight into how TLQP-62 modulate adult hippocampal neurogenesis and may suggest a mechanism for the antidepressant-like effects of VGF-derived peptides that we and others have reported (Hunsberger et al., 2007; Thakker-Varia et al., 2007, 2010).

Neurogenesis in the adult rodent hippocampus is a highly systematic process involving proliferation, differentiation, and migration that is controlled by various factors (Palmer et al., 1997). New granule cell neurons arise from the neural progenitors of the subgranular zone of the dentate gyrus (Song et al., 2002) and many factors including both intracellular and extracellular contribute towards the different stages of neurogenesis. Trophic factors such as FGF-2 (Zhao et al., 2007) and NT-3 (Shimazu et al., 2006) along with growth factors such as IGF-1 (Aberg et al., 2000) and VEGF (Jin et al., 2002) have been shown to have an effect on different phases of neurogenesis in the dentate gyrus. We have previously shown that neuropeptide TLQP-62 increases adult neurogenesis (Thakker-Varia et al., 2007) and in this study using markers of different stages of proliferation we have deciphered the exact stage of neuronal development at which TLQP-62 has the most effect. Specifically, TLQP-62 increases the number of Type 2a (undetermined) cells. Different neurogenic stimuli have been shown to affect the dividing cells at different stages of neuronal development. Type 1 cells are the stem cells and generally do not respond to many stimuli. On the other hand Type 2 cells do respond to chemical stimuli such as antidepressants, as well as physiologic activities such as environmental enrichment and exercise (Kronenberg et al., 2003).

The effects of TLQP-62 on neurogenesis are thus similar to what is reported for antidepressant fluoxetine and serotonin (5-HT), both of which increase the proportion of undetermined Type 2a NPCs marked by Nestin+ and DCX-immunostaining and lacking the radial glial-like morphology (Boku et al., 2011; Encinas et al., 2006). The effect of TLQP-62 is also similar to the effect of exercise, which stimulates the division of both Type 2a and Type 2b cells (Kronenberg et al., 2003; Steiner et al., 2008). These findings support our theory that VGF-derived peptides affect a specific neurogenesis pathway and act as antidepressant-like agents (Hunsberger et al., 2007; Thakker-Varia et al., 2010) since both fluoxetine and exercise promote neurogenesis and have antidepressant-like actions (Engesser-Cesar et al., 2007; Huang et al., 2012). In contrast, kainic acid, a compound that induces seizures and subsequently results in aberrant neurogenesis, but does not have antidepressant-like activity, is known to promote Type 3 (determined migratory) NPCs over the earlier stages in its differentiation pathway (Jessberger et al., 2005; Steiner et al., 2008). Thus, our results suggest that VGF-derived peptides, which are known to have antidepressant effects in animal models (Hunsberger et al., 2007; Thakker-Varia et al., 2007, 2010), have a similar mechanism in promoting neurogenesis as "classical" antidepressants.

In this study, the mechanism of TLQP-62-induced proliferation was dissected using NPCs *in vitro*. We detected equivalent increased proliferation of NPCs by TLQP-62 on both coated plates as well as a bed of hippocampal cultures suggesting that the effect of TLQP-62 on NPC proliferation is cell autonomous and does not require factors provided by other cells in the hippocampus. In contrast, there was no significant effect of TLQP-62 on differentiation of NPCs on either type of substrate, consistent with the *in vivo* data that TLQP-62 promotes early phases of neurogenesis rather than differentiation and indicating that non-cell autonomous factors are not sufficient to induce differentiation by TLQP-62. The positive control, KCl, was expected to significantly enhance differentiation relative to the untreated controls (Deisseroth et al., 2004), which it only did on the polyornithine plus laminin coated dishes. It is believed that KCl failed to enhance differentiation when NPCs were plated on a bed of hippocampal neurons in our study because the prolonged exposure to KCl could have had excitotoxic effects on the mature hippocampal neurons which in turn may have affected the health of the NPCs.

Primary adult hippocampal neural progenitor cells were used to demonstrate that the effects of TLQP-62 are not

specific to stem cell lines. We observed a robust effect of TLQP-62 on proliferation of primary adult hippocampal progenitors. The primary cells, once dissociated from the neurospheres and plated on a coated substrate in the absence of FGF, show a reduced baseline proliferation relative to the stem cell line. The plated cells are perhaps very sensitive and responsive to growth factors which likely explains the higher fold effect of TLQP-62 in the primary cells relative to the cell line. The studies using TLQP-62 peptide in this study are gain-of-function experiments which could cause a pharmacologic peptide response. To address this possibility, scrambled peptide with the same amino acid composition of TLQP-62, PEHN-62, was used as a control and we observed no response of the cells to the scrambled peptide suggesting that the effects are specific to TLQP-62. Future experiments using TLQP-62 knockdown approaches will confirm the effects are specific to TLQP-62.

To further delineate the role of TLQP-62 in proliferation, we examined the cell cycle status of the cells treated with the neuropeptide. D-type cyclins control the transition from G1 to S (Sherr, 1994). D-type cyclins are thought to be a molecular link between the extracellular environment and the cell cycle machinery since they are synthesized in response to mitogens and their expression rapidly declines when mitogens are withdrawn (Matsushime et al., 1994). D-type cyclins have been shown to be regulated by other neuropeptides that affect proliferation in normal as well as neoplastic cells in the body (de Mendonca et al., 2013; Fernandez-Martinez et al., 2009; Song et al., 2003). In the nervous system, neuropeptides such as pituitary adenylate cyclase-activating polypeptide (PACAP) and extracellular signaling molecule, Wnt7a stimulate Cyclin D expression and regulate cerebral cortical proliferation (Qu et al., 2013; Yan et al., 2013). Moreover, Cyclin D2 knockout mice have impaired adult hippocampal neurogenesis (Ansorg et al., 2012; Kowalczyk et al., 2004) whereas overexpression of Cyclin D1 causes an expansion of the neural progenitor cells in the adult mouse hippocampus (Artegiani et al., 2011). Our data demonstrate that *cyclin D1 and D2* mRNA levels are upregulated in response to TLQP-62 treatment of NPCs but that scrambled control peptide PEHN-62 has no effect on *cyclin D* expression. Thus one mechanism by which TLQP-62 regulates neurogenesis may be by altering the expression of proteins which control the cell cycle machinery.

We now demonstrate that TLQP-62-enhanced synaptic activity (Alder et al., 2003; Bozdagi et al., 2008) is required for the effect of the neuropeptide on neurogenesis of NPCs. Our findings are consistent with studies which found that electrical excitation drives NPC neurogenesis in the adult hippocampus (Bruel-Jungerman et al., 2006; Deisseroth et al., 2004; Walker et al., 2008). The inhibition of TLQP-62-induced proliferation by TTX is supported by the fact that NPCs possess K^+ and Na^+ channels (Leng et al., 2009; Mistry et al., 2002). However, while KCl promotes neurogenesis in a Ca^{2+}-dependent manner (Deisseroth et al., 2004), TLQP-62 does not require Ca^{2+} channel activation. The difference in findings may relate to the fact that TLQP-62 promotes proliferation while KCl enhances the differentiation phase of neurogenesis.

The presence of neurotransmitter receptors in NPCs including GABA, AMPA and NMDA receptors has also been documented by expression analysis (Kitayama et al., 2004) as well as electrophysiological studies in the presence of pharmacological agents (Mistry et al., 2002). The specific receptors we identified as required for TLQP-62-induced neurogenesis include mGluR5 and NMDA, consistent with reports demonstrating the role of glutamate receptors in neurogenesis. A recent study demonstrates that mGluR5 mutant mice have reduced numbers of cells expressing markers of proliferation and differentiation (Xiao et al., 2013) and that antagonists to metabotropic glutamate receptors inhibit neurogenesis *in vivo* (Baskys et al., 2005; Xiao et al., 2013). Together these studies suggest a positive role for mGluR5 in neurogenesis although opposite results have been reported for Group II metabotropic receptors (Feng et al., 2011; Yoshimizu and Chaki, 2004).

The role of the NMDA receptor in promoting neurogenesis has also been documented. Treatment of NPCs with NMDA increased the number of proliferating cells in the adult dentate gyrus (Joo et al., 2007). Furthermore, the regulation of NMDA receptor expression is positively correlated with neurogenesis and *vice versa* (Kalev-Zylinska et al., 2009; Marx et al., 2011; Ren et al., 2013; Sharma et al., 2012). Moreover, NMDA receptor activation is required for both basal neurogenesis and enhanced neurogenesis of NPCs elicited by KCl (Deisseroth et al., 2004) and downregulation of NMDA receptor expression mediates impaired neurogenesis in the hippocampus of knockout mouse models (Sha et al., 2013). Finally, NMDA subunit receptor deletion from adult-born neurons impairs a neurogenesis-dependent form of LTP (Kheirbek et al., 2012). While all of these studies suggest a positive role of NMDA in promoting neurogenesis, other studies have shown that NMDA receptor activation inhibits neurogenesis (Cameron et al., 1995; Nacher et al., 2001), however these effects may depend on NMDA concentration, age of cells and stage of development.

Our data suggest that PKD phosphorylation is a key downstream event after TLQP-62-induced activation of mGluR5 receptors in NPCs. Our data also suggest that this kinase is required for TLQP-62-induced proliferation of NPCs. These findings are supported by a study, which shows that activation of mGluR5 induces phosphorylation of PKD in hippocampal neurons (Krueger et al., 2010). PKD, which is a serine threonine kinase is considered to belong to the Ca^{2+}/calmodulin dependent kinase superfamily (Valverde et al., 1994). PKD has been implicated in promoting DNA synthesis and cell proliferation in several cellular systems and is involved in vascular endothelial growth factor (VEGF) mediated proliferation (Wong and Jin, 2005). Consistent with PKD's promotion of proliferation, the kinase also has anti-apoptoic properties in tumor cells (Trauzold et al., 2003). Similarly, in addition to its effect on proliferation, VGF has been shown to have anti-apoptotic activity. A shorter VGF peptide, TLQP-21, was shown to prevent cerebellar granule cell death induced by serum and potassium deprivation suggesting a trophic-like activity for VGF. The effect of TLQP-21 in survival of cerebellar granule cells required PKC activation (Severini et al., 2008) suggesting that the same VGF signaling mechanisms may promote proliferation and prevent apoptosis. Transcriptional changes regulated downstream of TLQP-62's activation of mGluR5 *via* PKD involved in proliferation and survival remain to be elucidated.

We identified CaMKII as a downstream target of NMDA receptors activated in NPCs following TLQP-62 treatment. CaMKII can associate with NMDA receptors and ultimately sustain its activation state (Bayer et al., 2001). Supporting

our novel findings for a role of CaMKII in neurogenesis is a study showing that mouse mutants for CaMKII have impairments in maturation of granule cells in the dentate gyrus (Yamasaki et al., 2008). A recent study demonstrates that olfactory bulbectomized mice treated with a steroid receptor agonist that activates CaMKII have increased neurogenesis as well as LTP (Moriguchi et al., 2013). The mechanism by which CaMKII induces TLQP-62-mediated proliferation following NMDA receptor activation has yet to be explored.

Recently, two receptors for TLQP-21 have been described (Chen et al., 2013; Hannedouche et al., 2013). Both of these receptors C3aR1 and gC1qR are complement receptors and are thought to be involved in the role of TLQP-21 in neuropathic pain (Chen et al., 2013) and metabolism (Hannedouche et al., 2013). It has not yet been tested whether TLQP-62 binds to either of these receptors or whether they are expressed in the hippocampus. In this study, we explored the possible interactions between TLQP-62 with glutamate receptors as well as the BDNF receptor to yield novel insights into the signaling pathways of TLQP-62. We found that TLQP-62 requires activation of Trk receptors to mediate its effect on neurogenesis. Moreover, TLQP-62 induces phosphorylation of TrkB, albeit with a slower time course and to a lesser extent than BDNF. This suggests that TLQP-62 may not activate TrkB directly, but is perhaps inducing the release of BDNF, which in turn is binding to its receptor TrkB and promoting glutamate release. Alternatively, TLQP-62 could transactivate TrkB independently of BDNF as previously shown for adenosine and PACAP (pituitary adenylate cyclase-activating polypeptide) (Lee and Chao, 2001; Lee et al., 2002a,b). Our findings are supported by a previous study, which suggests that TrkB may be downstream of the effect of TLQP-62 on synaptic activity. Specifically, the transient potentiation of activity by TLQP-62 peptide could be blocked by the BDNF scavenger TrkB-Fc, the Trk tyrosine kinase inhibitor K252a and the tPA inhibitor tPA STOP (Bozdagi et al., 2008).

The role of BDNF and its receptor TrkB in neurogenesis has been explored. Overexpression or infusion of BDNF in the adult rat results in newly generated cells (Benraiss et al., 2001; Pencea et al., 2001; Scharfman et al., 2005) and BDNF is required for enhancement of hippocampal neurogenesis (Lee et al., 2002a,b; Rossi et al., 2006). Although some studies suggest that BDNF promotes the survival rather than proliferation or differentiation of new neurons (Bath et al., 2008; Sairanen et al., 2005), TrkB deletion specifically in progenitor cells of the dentate gyrus results in impaired neurogenesis induced by antidepressant treatment, suggesting that proliferation of progenitor cells is mediated by TrkB signaling. Deletion of TrkB in differentiated cells of the dentate gyrus, on the other hand, had no effect on the number of neurons (Li et al., 2008). A possible explanation as to why some studies have found that BDNF affects proliferation (Li et al., 2008) while others have found it to only affect survival (Sairanen et al., 2005) is the use of different promoters to delete TrkB affected different cell populations (Banasr and Duman, 2008). Our studies suggest that TrkB may be playing a role in TLQP-62-induced proliferation of NPCs.

We have previously shown that TLQP-62 expression is induced by factors that have antidepressant activity. Conversely, *VGF* is downregulated in rodent models of depression (Thakker-Varia et al., 2007). Along with others, we have demonstrated that infusion of VGF-derived peptides in the hippocampus of mice results in antidepressant-like outcomes (Hunsberger et al., 2007; Thakker-Varia et al., 2010). We have also shown that *VGF* is downregulated in human bipolar disorder and acts as a mood stabilizer in models of mania. Moreover, *VGF* levels are downregulated in leukocytes of depressed patients but are restored in response to antidepressant treatment (Cattaneo et al., 2010). BDNF has also been implicated in depression (Castren et al., 2007; Duman and Monteggia, 2006). Antidepressant agents activate TrkB (Rantamaki et al., 2007) and local infusion of BDNF or overexpression of TrkB receptor in the brain mimics antidepressant effects in animal models (Koponen et al., 2005; Shirayama et al., 2002; Siuciak et al., 1997). On the other hand, BDNF signaling is essential for the behavioral effects of the drugs (Monteggia et al., 2004; Saarelainen et al., 2003). Thus, decreased levels of VGF and BDNF contribute to depression and increased levels *via* antidepressant treatment result in enhanced neurogenesis in the hippocampus. Our data showing activation of TrkB by VGF-derived peptide TLQP-62 support a possible link between neuropeptides and neurotrophins in antidepressant actions.

The ultimate identification of the TLQP-62 receptor and other interacting proteins will reveal the other players involved in TLQP-62-mediated neurogenesis and give additional insight into the role that synaptic activity has on that process. Stem cells in the hippocampus have been suggested to have roles in learning and memory as well as psychiatric disorders. Thus understanding which external factors influence the production and differentiation of new neurons in the dentate gyrus and the mechanism of their action is critical for future studies which may manipulate this stem cell population for therapeutic purposes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.03.005.

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